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Brief Definitive Report

Class I Dependence of the Development of CD4$^+$CD8$^-$NK1.1$^+$ Thymocytes

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Summary

A small subset of functionally active CD4$^+$CD8$^-$ thymocytes express the NK1.1 marker, as do most CD4$^-$CD8$^-$NK1.1$^+$ thymocytes. Previous studies have failed to implicate a role for major histocompatibility complex (MHC) or related molecules in the selection of the CD4$^+$CD8$^-$NK1.1$^+$ subset. We report here that the development of most of these cells is sharply reduced in class I–deficient mice, but not in class II–deficient mice. Hence, some CD4$^+$ T cells are class I dependent and not class II dependent. Unlike conventional T cells, however, the development of NK1.1$^+$ thymocytes in both the CD4$^+$CD8$^-$ and CD4$^-$CD8$^-$ subsets is dependent on class I MHC expression by hematopoietic cells and not thymic epithelial cells. We propose that these populations are selected by nonpolymorphic class Ib or CD1 molecules.

Little is known concerning the development of two recently defined TCR-α/β^+ T cell subsets, TCR-α/β^+ CD4$^-$CD8$^-$NK1.1$^+$ cells and CD4$^+$CD8$^-$NK1.1$^+$ cells. These populations represent small subsets of late-arising thymocytes and peripheral T cells, which are characterized by a mature phenotype (heat-stable antigen [HSA$^-$]) and functional capabilities (1–9). A relationship between these two subsets is suggested by the shared expression of NK1.1 and Ly6C, as well as the expression of relatively low cell surface levels of TCR-α/β receptor by most cells in both subsets. Furthermore, both subsets secrete relatively high levels of IL-4 and IFN-γ upon stimulation with immobilized anti-TCR mAbs, unlike naive conventional CD4$^+$CD8$^-$ thymocytes which produce predominantly IL-2 (8–10).

Interestingly, these subsets exhibit a strongly increased frequency of expression of Vβ8 genes, particularly Vβ8.2, compared with conventional TCR-α/β^+ T cells (1–3, 6, 8, 9). Previous studies have addressed whether CD4$^-$CD8$^-$Vβ8$^+$ cells are subject to MHC-dependent positive and negative selection in their development, and have generated conflicting results. However, we recently demonstrated that these cells are sharply diminished in class I–deficient mice, suggesting that class I molecules play a role in their selection and/or expansion (11). Based on the similarities of CD4$^+$CD8$^-$NK1.1$^+$ cells and CD4$^-$CD8$^-$TCR-β^+ cells, we have investigated the development of CD4$^+$CD8$^-$NK1.1$^+$ thymocytes. Here we report that the CD4$^+$CD8$^-$NK1.1$^+$ population is largely dependent on the expression of class I molecules by hematopoietic cells, but not on the expression of class II molecules.

Materials and Methods

Mice. C57BL6/J (B6) and BALB/cByJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). β_2 microglobulin (β_2m) mutant mice ($\beta_2m^{-/-}$) (12) had been backcrossed five times to the B6 strain. Class II–deficient mice harboring a mutated Aβ^b gene (13), had also been backcrossed five times to B6, and were purchased from GenPharm International (Mountain View, CA) or were gifts of Dr. Laurie Glimcher (Harvard Medical School, Boston, MA) or Dr. Virginia Papaioannou (Tufts Medical School, Boston, MA). Double mutant A$\beta^{-/-}$, $\beta_2m^{-/-}$ mice were bred from the single mutant animals. Mice were used between 8–10 wk of age.

Antibodies. H57.597-2.1 (anti-TCR-β [14]), F23.1 (anti-Vβ8.1, 8.2, 8.3 [15]), and PK136 (anti-NK1.1 [16]), purified from culture supernatants, were biotinylated or fluoresceinated. RM4.4-PE (anti-CD4; PharMingen, San Diego, CA), M1/69-FITC (anti-HSA; PharMingen), 53.67.2-Biotin (anti-CD8α; PharMingen) and 53.67.2-Red613 (anti-CD8α; GIBCO BRL, Gaithersburg, MD) were purchased. Second step reagents included tricolor-streptavidin (Tri-SA) (Caltag Laboratories, S. San Francisco, CA) and APC-streptavidin (APC-SA) (Molecular Probes, Inc., Eugene, OR).

Cell Preparation and Flow Cytometry. Thymocytes were depleted of CD8$^+$ T cells by double antibody and complement treatments (11). The resulting population was routinely >99% devoid of brightly staining CD8$^+$ cells. The CD8-depleted cells (10^6) were incubated in the first step with CD4-PE, a biotinylated reagent and a fluoresceinated reagent, and in the second step with APC-SA. 5×10^4 total events were analyzed. Gated CD4$^+$ or CD4$^-$ cells were analyzed on a dual laser modified FACS IV® (Becton Dickinson & Co., Mountain View, CA). For four-color analysis, unfractionated thymocytes were stained in the first step with anti-CD4-PE, anti-CD8-Red613, NK1.1-FITC, and anti-TCR-β–biotin, and in the second step with Tri-SA. 3×10^5 events were analyzed

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on an XL flow cytometer (Coulter Corp., Hialeah, FL), using forward and side scatter to exclude dead cells and debris.

Production of Irradiation Fetal Liver Chimeric Mice. Chimeras were produced by inoculating irradiated 8-wk-old mice (980 rad from a ^{137}Cs source) with 1.5 × 10^7 E16 fetal liver cells (11), and were analyzed at 8 wk after reconstitution. Recipients were depleted of NK1.1$^+$ cells before irradiation and reconstitution by inoculation with 200 μg i.p. of anti-NK1.1 mAb (PK136), in order to prevent NK-mediated rejection of the fetal liver transplants (17). β_2m$^+$ recipients and donors were inbred B6 mice.

Results

Phenotypic Analysis of NK1.1$^+$ Defined Thymocyte Populations. 7–15% of double-negative thymocytes expressed NK1.1, of which 50–75% also expressed TCR-β (Fig. 1 *A*). 4–7% of CD4$^+$CD8$^-$ thymocytes expressed NK1.1, and essentially all of these cells were TCR-β^+, though the level of TCR-β on these cells was lower than seen on "conventional" CD4$^+$CD8$^-$NK1.1$^-$ thymocytes (Fig. 1 *B*). The large majority of both types of NK1.1$^+$ thymocytes had a mature HSA$^-$ phenotype, half or more expressed Vβ8 (Fig. 1), and most were also Ly6C$^+$ (data not shown), corroborating the extensive overlap of the NK1.1$^+$ subsets with the TCR-α/β^+ and Ly6C$^+$ thymocyte populations (18–20). Closer scrutiny of the NK1.1$^+$ "CD4$^-$CD8$^-$" and "CD4$^+$CD8$^-$" thymocyte populations revealed that these cells express low but significant levels of CD8, as shown by the slightly increased CD8 staining of these cells relative to CD8$^-$ NK1.1$^-$ thymocytes (Fig. 1 *C*). Therefore, cells in the NK1.1$^+$ thymocyte populations will be referred to hereafter as CD4$^-$CD8^{lo}NK1.1$^+$ or CD4$^+$CD8^{lo}NK1.1$^+$ thymocytes.

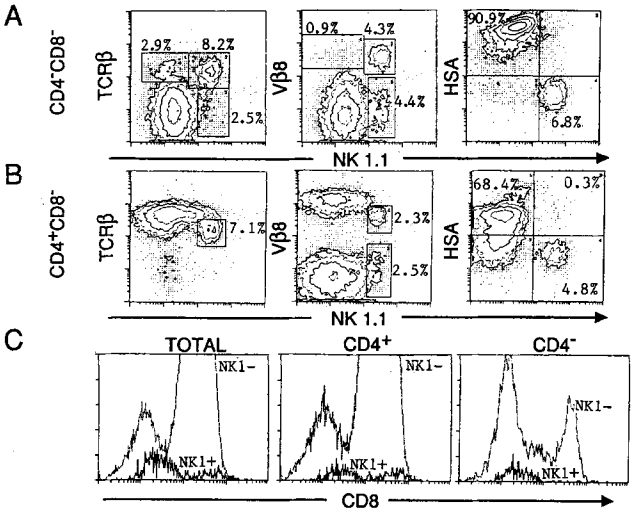

Figure 1. Phenotypic analysis of CD4$^-$CD8$^-$ and CD4$^+$CD8$^-$ thymocytes from B6 mice. For *A–C*, thymocytes were depleted of CD8$^+$ cells and gated CD4$^-$ (CD4$^-$CD8$^-$) thymocytes (*A*) or CD4$^+$ (CD4$^+$CD8$^-$) thymocytes (*B*) were analyzed by three-color staining. (*C*) Expression of CD8 on NK1.1$^+$ vs. NK1.1$^-$ thymocytes determined by three-color analysis of total thymocytes (*left*), gated CD4$^+$ thymocytes (*middle*), and gated CD4$^-$ thymocytes (*right*).

Selection of NK1.1$^+$ Thymocytes by MHC Molecules. We compared thymocytes from wild-type mice with those from β_2m$^-$ (class I–deficient) mice, Aβ^- (class II–deficient) mice, or β_2m$^-$Aβ^- (class I– and class II–deficient) mice (Fig. 2 and Table 1). The number of thymocytes and the proportion of double-negative cells did not differ significantly among these mice (Table 1 and data not shown). The frequency of CD4$^+$CD8^{lo}NK1.1$^+$ thymocytes was reduced in class I–deficient mice by nearly 90% compared with wild-type mice, whereas class II deficiency did not diminish the CD4$^+$CD8^{lo}NK1.1$^+$ population; if anything there was a slight increase in this population (Fig. 2 and Table 1). Mice deficient for both class I and class II expression had low frequencies of TCR-β^+CD4$^+$CD8^{lo}NK1.1$^+$ cells, similar to or slightly higher than observed in mice deficient for class I alone.

The CD4$^-$CD8^{lo}NK1.1$^+$TCR-β^+ population was reduced by 70–75% in class I–deficient mice or in mice deficient for both class I and class II (Fig. 2 and Table 1). A smaller reduction in total CD4$^-$CD8^{lo}NK1.1$^+$ cells was observed due to the existence in some mice of a substantial population of CD4$^-$CD8^{lo}NK1.1$^+$ cells that does not express TCR-β and that is mostly unaffected by class I deficiency (Table 1 and data not shown). Class II deficiency by itself had little or no effect on the frequency of CD4$^-$CD8^{lo}NK1.1$^+$ thymocytes (p >0.2 by Student's t test) (Fig. 2 and Table 1). This data contrasts with our previous results, which suggested that the frequency of CD4$^-$CD8$^-$TCR-α/β^+ cells was modestly decreased, by ~40%, in class II–deficient mice (11). In the earlier study, TCR-β^+ "double-negative" thymocytes were enriched by anti-CD4 and C treatment. This treatment may have depleted some of these cells, which in class II–deficient mice appear to express low levels of CD4 (data not shown). In conclusion, class I but not class II deficiency causes a sharp reduction in NK1.1$^+$ thymocytes of the CD4$^+$CD8^{lo} and CD4$^-$CD8^{lo} phenotypes.

Development of NK1.1$^+$ Thymocyte Populations Is Dependent on Class I Expression by Hematopoietic Cells. To investigate the contribution of class I expression by hematopoietic cells or thymic epithelial cells to the development of NK1.1$^+$ T cell populations, we constructed fetal liver chimeras between class I deficient (β_2m$^-$) and normal mice. In these chimeras the fetal liver donor contributes most of the hematopoietic cells and the host contributes the thymic epithelial cells. As we had previously found for Vβ8$^+$CD4$^-$CD8$^-$ thymocytes (11), both the TCR-β^+CD4$^-$CD8^{lo}NK1.1$^+$ and the CD4$^+$CD8^{lo}NK1.1$^+$ populations were strongly diminished in β_2m$^-$ → β_2m$^+$ fetal liver chimeras, but not in β_2m$^+$ → β_2m$^-$ chimeras (Fig. 3). The β_2m$^-$ → β_2m$^-$ and β_2m$^+$ → β_2m$^+$ control chimeras yielded NK1.1$^+$ populations similar in frequency to unmanipulated wild-type and β_2m$^-$ mice, respectively. The results indicate that the development of both of these NK1.1$^+$ populations is dependent primarily on class I expression by hematopoietic cells rather than by thymic epithelial cells. Conventional CD8$^+$ T cells, in contrast, were diminished in β_2m$^+$ → β_2m$^-$ chimeras

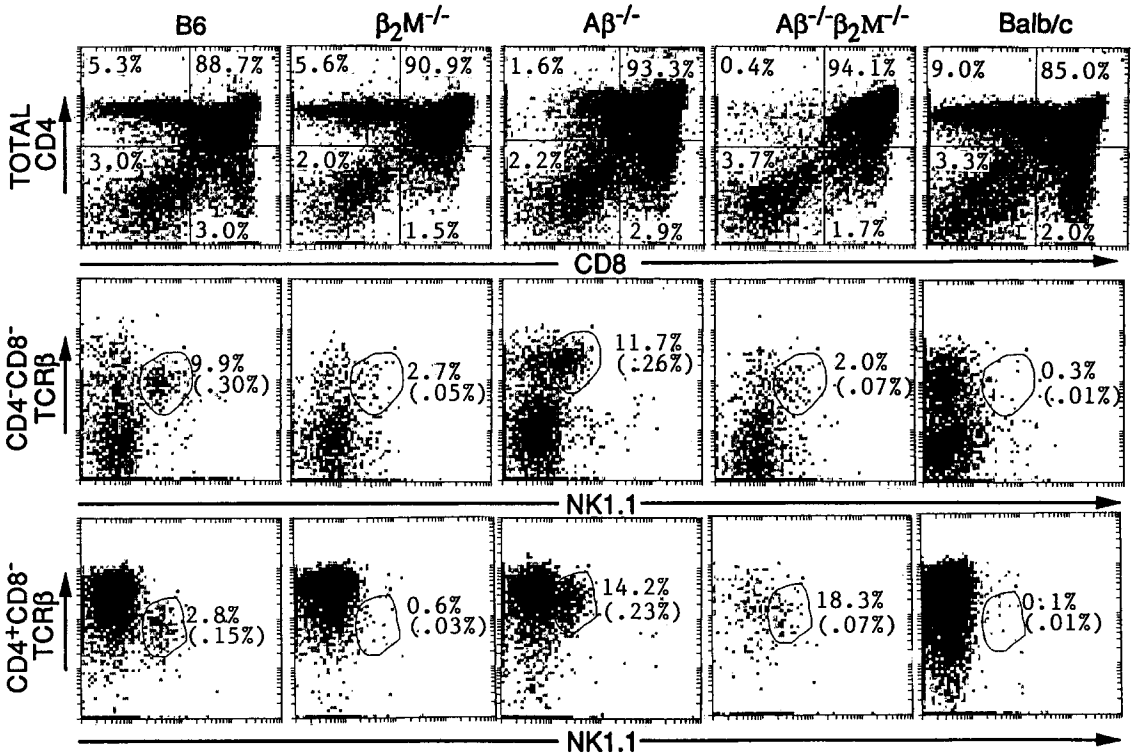

Figure 2. NK1.1 and TCR-β expression on thymocyte subsets from B6, $\beta_2m^{-/-}$ (class-I-deficient), $A\beta^{-/-}$ (class II deficient), and $A\beta^{-/-}\beta_2m^{-/-}$ (class I and class II deficient) mice. The top row depicts CD4 vs. CD8 staining for unfractionated thymocytes. NK1.1 and TCR-β expression on gated $CD4^-CD8^{lo}$ thymocytes (*middle*) and gated $CD4^+CD8^{lo}$ thymocytes (*bottom*) was determined by four-color analysis. The numbers represent the percentage relative to the population under analysis, whereas the numbers in parentheses represent the percentage relative to the total thymocyte population. BALB/c mice are $NK1.1^-$, and these thymocytes thus serve as a control for the specificity of the NK1.1 reagent.

and not in the $\beta_2m^- \rightarrow \beta_2m^+$ chimeras (data not shown [11, 17]).

Discussion

The primary conclusion of the present study is that a subset of $CD4^+$ T cells, characterized by NK1.1 expression, is dependent for its development on the expression of class I molecules but not on the expression of class II molecules. Furthermore, the development of these cells depended on class I expression by hematopoietic cells and not by thymic epithelial cells.

Recent studies describe a subset of $CD4^+CD8^{lo}$ thymocytes presumed to be intermediates in the development of conventional $CD4^+$ T cells. The cells were present in class II–deficient mice but not in class I and class II double–deficient

Table 1. *Frequencies of NK1.1-defined Thymocyte Populations in MHC-deficient Mice*

Strain	Thymic cell no. (10^6)	TCR-β^+NK1.1$^+$ (Percent of $CD4^-CD8^-$)	Vβ8$^+$NK1.1$^+$ (Percent of $CD4^-CD8^-$)	Percent NK1.1$^+CD4^-CD8^-$ (Percent of thymus)	Percent NK1.1$^+CD4^+CD8^-$ (Percent of thymus)
B6	150 ± 18	7.4 ± 1.3	5.4 ± 1.9	0.33 ± 0.03	0.26 ± 0.02
β_2m^-	176 ± 20	2.0 ± 0.5*	0.4 ± 0.2	0.19 ± 0.04*	0.03 ± 0.01*
$A\beta^-$	178 ± 31	5.5 ± 1.0	ND	0.40 ± 0.12	0.42 ± 0.09*
$\beta_2m^-A\beta^-$	133 ± 19	2.6	ND	0.25 ± 0.03	0.08 ± 0.01*

Values represent means and standard errors of three to nine individual determinations, except in the instance where no standard error is shown, which represents a single determination. All mice were 8–10 wk old. For the second and third columns, CD8-depleted thymocytes, gated on the $CD4^-$ population, were analyzed. Columns 4 and 5 represent determinations on unfractionated thymocytes by three-color analysis.
*$p < 0.02$ by Student's t test compared with the values for wild-type (B6) mice.

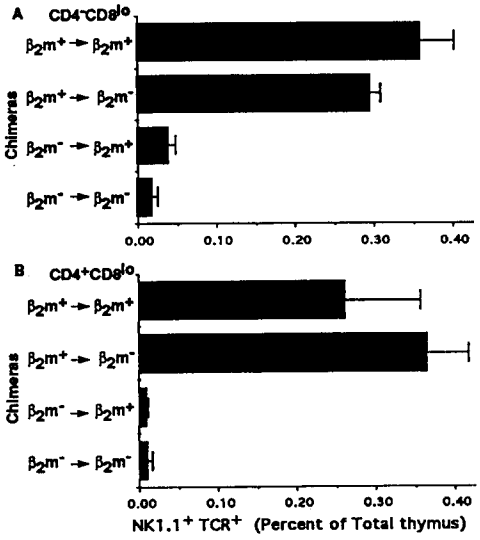

Figure 3. Class I expression on hematopoietic cells is necessary for the appearance of NK1.1$^+$CD4$^-$CD8^{lo} and NK1.1$^+$CD4$^+$CD8^{lo} cells. Fetal liver chimeras were analyzed at 8 wk post reconstitution. Gated CD4$^-$ CD8$^{lo/-}$ (*A*), and CD4$^+$CD8$^{lo/-}$ (*B*) thymocytes were analyzed for NK1.1 and TCR expression by four-color staining. Separate three-color staining analysis with an anti-class I (K^b) reagent, anti-CD4, and anti-CD8 showed that in all of the chimeras analyzed, >95% of CD4$^-$ CD8$^{lo/-}$, or CD4$^+$CD8$^{lo/-}$ thymocytes were of donor origin.

mice (21). A two-step positive selection model was proposed in which initial TCR engagement by class I or class II MHC leads to the stochastic downregulation of CD8 or CD4. Subsequently, successful selection may occur if the MHC specificity of the retained coreceptor matches that of its TCR. The CD4$^+$CD8^{lo}NK1.1$^+$ thymocytes are unlikely to correspond to these putative CD4$^+$CD8^{lo} intermediate cells for several reasons: (*a*) the CD4$^+$CD8^{lo}NK1.1$^+$ cells arise in ontogeny significantly after the appearance of fully mature conventional CD4$^+$CD8$^-$ T cells (6, 7); (*b*) they have a fully mature phenotype, and produce cytokines when stimulated with anti-TCR mAb (8–10); and (*c*) they have a distinct repertoire with high usage of Vβ8. Furthermore, it seems unlikely that an intermediate in CD4$^+$ cell differentiation would be diminished by class I deficiency but not by class II deficiency, as shown here for the CD4$^+$CD8^{lo}NK1.1$^+$ thymocytes. It appears more likely that the putative intermediate corresponds to the CD4$^+$CD8^{lo}NK1.1$^-$ cells, which are the majority of CD4$^+$CD8^{lo} cells in class II–deficient mice (Fig. 2), and which express relatively high levels of TCR-β, similar to the levels found on mature CD4$^+$CD8$^-$ cells (Fig. 2). The CD4$^+$CD8^{lo}NK1.1$^-$ cells are nearly absent in the class I$^-$class II$^-$ thymus (Fig. 2 and reference 21), but the available methods preclude a determination of whether they are present in the class I$^-$ thymus.

It was initially suggested that the production of IL-4 and IFN-γ by thymic CD4$^+$CD8$^-$ cells might be a consequence of recent positive selection of conventional CD4$^+$ cells (10). However, direct evidence indicates that the CD4$^+$CD8^{lo} NK1.1$^+$ population is responsible for the high level of IL-4 and IFN-γ production by CD4$^+$ thymocytes from normal mice (9). Because the evidence suggests that NK1.1$^+$ thymocytes are probably not intermediates in the development of conventional CD4$^+$CD8$^-$ cells, and CD4$^+$CD8$^-$NK1.1$^-$ thymocytes reportedly fail to produce IL-4 and IFN-γ (9), it now appears unlikely that production of IL-4 and IFN-γ is a consequence of recent positive selection.

The use of class I deficient mice has allowed us to identify class I molecules as selecting elements for both NK1.1-defined populations. Because earlier studies as well as our own have failed to identify MHC polymorphisms affecting the NK1.1-defined populations, we suggest that the relevant class I molecule(s) may correspond to a nonclassical class I molecule, such as the class 1b or CD1 molecules, most of which are relatively nonpolymorphic (22). Selection by a highly specific class I molecule could also account for the Vβ8-skewing in these populations, especially as it has been reported that some class Ib molecules present a highly specific set of peptides (23).

Both NK1.1$^+$ populations studied here have characteristics of activated cells or memory cells, including high expression of CD44. It might therefore be suggested that the class I–dependent selection of these cells results from antigen-dependent expansion of the cells after their maturation, rather than representing class I–dependent maturation of the cells. The late appearance of the cells is consistent with this possibility. Furthermore, the high frequency of Vβ8 usage is reportedly not evident in the TCR-β^+CD4$^-$CD8^{lo} population at the earliest time the cells can be detected, but rather occurs gradually with time after birth (20). Finally, the observed dependence of the development of both NK1.1$^+$ thymocyte populations on class I molecules expressed by hematopoietic cells, but not thymic epithelial cells, is consistent with the possibility that the cells accumulate as a consequence of APC-dependent expansion (11). If the NK1.1$^+$ T cell populations are expanded by stimulation with antigen(s) associated with MHC class I, the nature of the responsible antigen remains an open question. One candidate is an antigen(s) associated with intracellular bacteria, which may represent common environmental antigens. Perhaps the NK1.1$^+$ T cells are specialized for recognition of common bacterial pathogens presented by class Ib molecules (23, 24).

Neither the CD4$^+$CD8^{lo}NK1.1$^+$ population nor the TCR-β^+CD4$^-$CD8^{lo}NK1.1$^+$ population is significantly diminished by class II deficiency. Furthermore, the appearance of these cells does not depend on normal class I expression by thymic epithelial cells in the β_2m$^+$ → β_2m$^-$ fetal liver chimeras. In contrast, the development of CD8$^+$ T cells is reduced by ~85% in such chimeras (17). Therefore, either the maturation of NK1.1$^+$ thymocytes involves a distinct positive selection mechanism compared to conventional α/β T cells, or the cells mature without positive selection and are subsequently selectively expanded by interaction with class I molecules or class I–bound antigens.

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